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14. ABSTRACT We developed an HPLC method to determine the amount of EGCG encapsulated in the nanoparticles. HPLC analysis showed that chitosan nanoparticles can efficiently entrap EGCG. Also, we developed a method to extract EGCG from the plasma with a recovery value of > 95%. We determined the effect of oral nanoEGCG on the growth of tumors in athymic nude mice implanted with 22Rv1 cells. Treatment with EGCG (40 mg/kg body wt.) and nanoEGCG (3 and 6 mg/kg body wt.) resulted in significant inhibition of tumor growth. In control group, the average tumor volume of 1,200 mm3 was reached in 32 days after tumor cell inoculation. At this time point, the average tumor volume was 514, 310 and 216 in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively. The average tumor volume of 1,200 mm3 was achieved 46, 53 and 60 days after tumor cell inoculation in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively. At 28 days post-inoculation, there was 36, 54 and 72% decrease in the secreted PSA levels in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively as compared to the control group.

15. SUBJECT TERMS
Nanochemoprevention; EGCG; nanoEGCG; tumor growth; athymic nude mice; prostate cancer.

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INTRODUCTION:

Among all natural agents being tested for their cancer chemopreventive properties green tea has shown promise in preclinical, epidemiological and initial clinical studies. However, absolute clinical success has been hampered due to issues related to bioavailability and toxicity. This study proposes a novel modality involving nanoencapsulation of EGCG for oral consumption designed for prevention and treatment of prostate cancer. Because most biological processes including those that are cancer-related occur at nano-scale, nanotechnology could serve as a potential tool for this unmet need (1). Because of their size range, nanoparticles are very suitable for manipulations at the molecular level, for example cell-receptor binding for site-selective targeting, and localization of encapsulated therapeutics for delivery. With an increasing number of nanoparticle formulations under review by the FDA, and exponentially increasing submission of patents for novel formulations, the outlook for nanoparticle systems in cancer therapy is promising. Nanotechnology will offer significant and increasing improvements in options not only for therapeutic interventions against malignancies but also for disease prevention, a concept that is being addressed through this study (1). In a recent study, we reported significant dose-advantage of polylactic acid-polyethylene glycol (PLA-PEG) encapsulated EGCG (*nanoEGCG*) over non-encapsulated EGCG. NanoEGCG had over ten-fold dose advantage for exerting its pro-apoptotic and anti-angiogenic effects (2).

Oral consumption is the most desired and acceptable form of delivery of chemopreventive agents. Further, one disadvantage of using PLA-PEG nanoparticles is their unstable nature in acidic environment and therefore is not recommended for oral consumption (3). To overcome this obstacle we have been successful in developing an oral formulation of nanoEGCG employing a naturally occurring polymer chitosan which we observed to result in a steady and sustained release of EGCG in the plasma of mice. Our uniquely formulated oral nanoEGCG was synthesized in a mild acidic condition by promoting the interaction of NH3 group present in chitosan with the phosphate group present in Adenosine 5′-tri-phosphate (ATP). Size distribution and zeta potential of chitosan based nanoparticles encapsulating EGCG was determined by using a Malvern zetasizer (Malvern Instrumentation Co., Westborough, MA). The size of the nanoparticles was found to be in the range of ~200-250 nm in diameter and zeta potential was found to be positive (+ive) as anticipated. The positive charge is an indication of superior muco-adhesive properties of the nanoformulation, which is a prerequisite for any oral formulation. This nano formulated green tea has a significant longer half-life compared to non-encapsulated EGCG. The central hypothesis to be tested in this proposal is that our uniquely formulated nanoEGCG suitable for oral consumption will result in enhanced and sustained availability of EGCG leading to a robust decrease in the effective concentration and prevention of cancer development in mouse models of prostate cancer (PCa).

BODY:

Synthesis of chitosan-based nanoparticles encapsulating EGCG:

Chitosan nanoparticles were synthesized in mild acidic conditions by promoting the interaction of the NH_3 group present in chitosan with the phosphate group present in Adenosine 5′-triphosphate. Briefly, 95 ml of a solution of EGCG (20 mg/ml of DI water) was added to 5 ml of 1% water-soluble chitosan and stirred for 1 hour. Next, 1 ml of a triphosphate disodium salt (20 mg/ml in DI water) was added drop by drop, with constant stirring. The entire solution was then sonicated for about 30 seconds using a probe sonicator, and allowed to stir for another 4 hours

(approx). This solution, containing EGCG nanoparticles, was dialyzed to remove the impurities and the free ECGC using a 100 kD cut-off dialysis membrane. This solution was then lyophilized to get the nanoformulation in powdered form. This was then redispersed in DI water for further use.

Size measurement by Dynamic Light Scattering (DLS):

Size distribution of chitosan-based nanoparticles encapsulating EGCG in aqueous dispersion was determined by using a Malvern zeta sizer (Malvern Instrumentation Co., Westborough, MA, USA). 2 ml of chitosan-based nanoparticles encapsulating EGCG were placed in a 4-sided, clear, plastic cuvette and analyzed directly at 25°C. The size of the nanoparticles was found to be less than 200 nm in diameter (Figure 1).

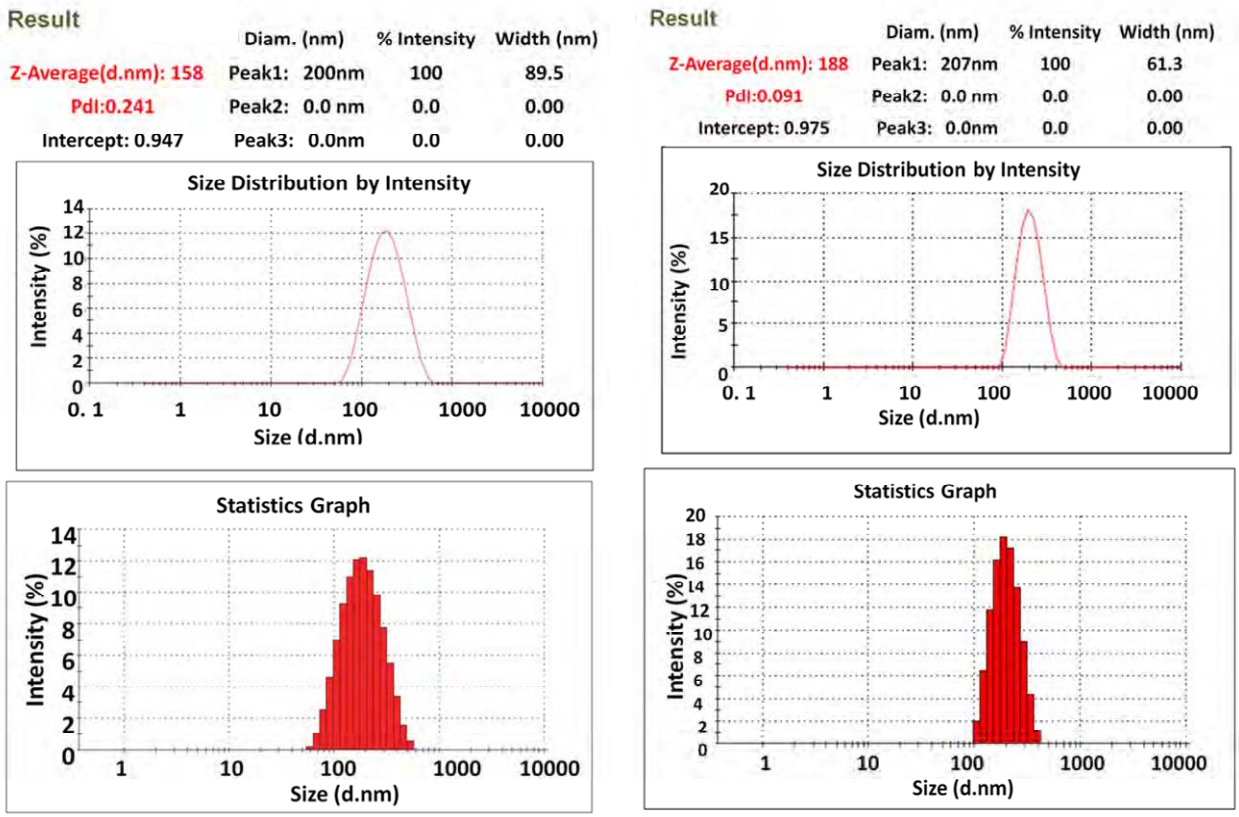


Figure 1: Size measurement by DLS of A) chitosan-based nanoparticles encapsulating EGCG, B) void chitosan nanoparticles.

Analysis of amount of EGCG in the nanoformulation: The amount of EGCG was determined by disintegrating the nanoparticles and measuring the EGCG by HPLC. This also helps in determination of the entrapment efficiency of EGCG in the nanoformulation. First, a calibration curve was obtained with a standard EGCG solution as shown in Figure 2.

The redispersed nanoparticles were disintegrated by adding an acetic acid solution. The entire solution was passed through a filter Millipore centrifugal device of 100 kD cut-off with the help of centrifugation, at around 6500 rpm for 15 minutes to separate the EGCG. The concentration of

the centrifugate containing EGCG was determined using LC/MS. The entrapment efficiency was determined by the following formula:

entrapment efficiency = $([EGCG]_f) / ([EGCG]_t) \times 100$
where $[EGCG]_f$ is the concentration of EGCG in the centrifugate and $[EGCG]_t$ is the theoretical concentration of EGCG (meaning total amount of EGCG added initially). Thus, it was found that entrapment efficiency of ECGC was around 75 %.

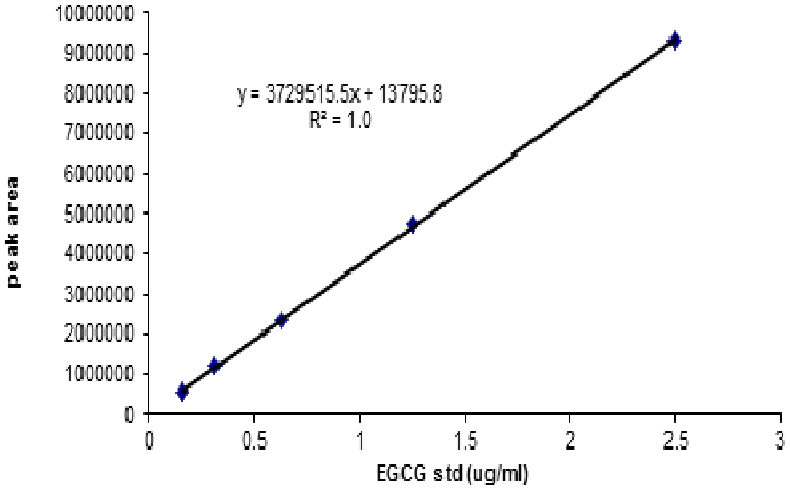
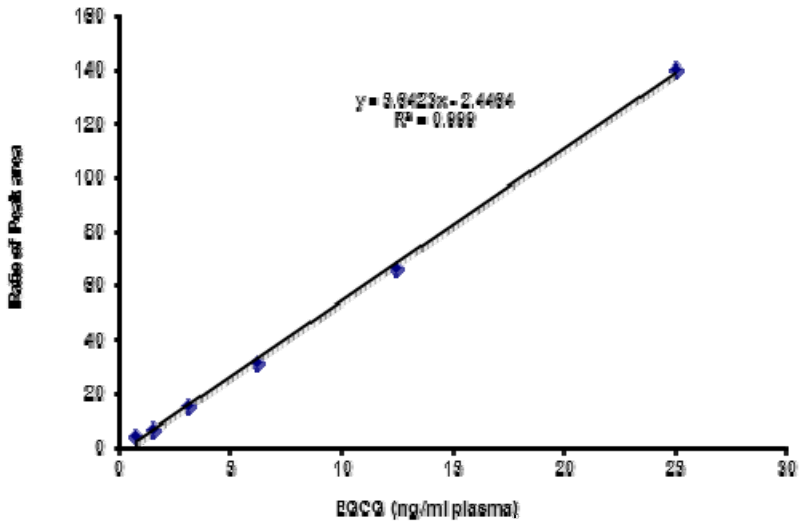


Figure 2: Calibration curve of standard EGCG solution.

LC/MS methods:
Calibration curves: Stock solutions of 0.5 mg/ml and 0.05 mg/ml of EGCG were prepared in 50% methanol (Figure 3). A stock solution of ethyl gallate of 5ug/ml was prepared in the same way in 50% methanol solution. Two calibration curves were obtained to achieve better accuracy. Figure 3A is lower concentration and Figure 3B is the higher concentration of EGCG.

(A)



(B)

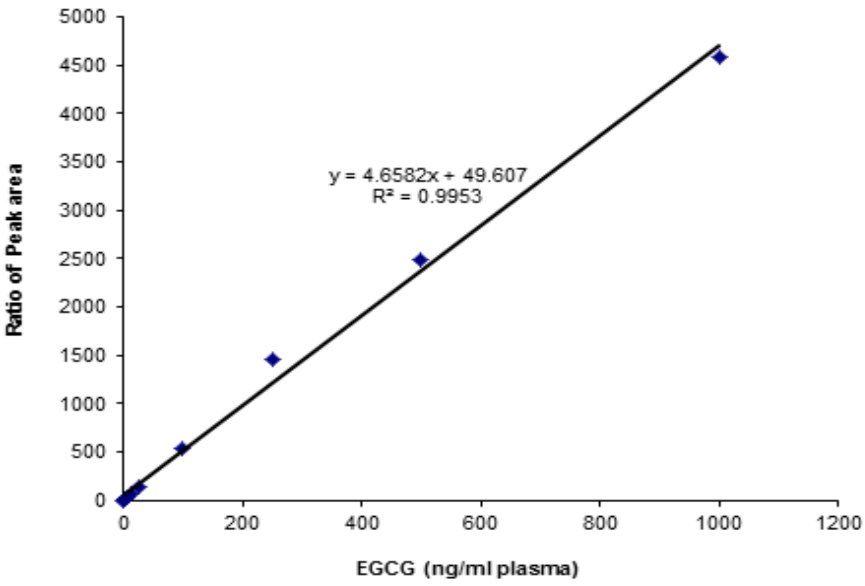


Figure 3: Calibration curve of EGCG spiked into plasma and measured with LC-MS/MS method.

Diluted human plasma (1:3 w/NS) was spiked with EGCG solution to prepare calibration samples ranging from 2 to 1000 ng/ml. 10 µl of the internal standard was added to each sample before extraction. For each sample, an aliquot of 100 µl of serum was extracted with ethyl acetate and acetonitrile, and the supernatant was dried. The residual was resuspended with 40% acetonitrile, and 25 µl was injected for the LC-MS/MS assay. An internal standard was used to normalize the amount of EGCG recovery from the plasma. It was found that the limit of detection (LOD) is 0.05 ng and the limit of quantification (LOQ) is 1 ng/ml in an amount of 80 µl.

- Shimadzu HPLC includes: LC 20AD pumps; SIL-20AC auto sampler, CTO-20AC column oven and CBM-20A communication bus module;
- Mass spectrometry: Applied Biosystems API 4000 triple quadrupole mass spectrometer, equipped with a Turbo IonSpray source ionizing in the negative mode;
- Isocratic chromatography in MRM mode was performed on a SunFire C18 column, kept at 40 ºC;
- m/z transitions of EGCG: 457/168.9;
- m/z transitions of ethyl gallate (internal standard): 168.9/124.9

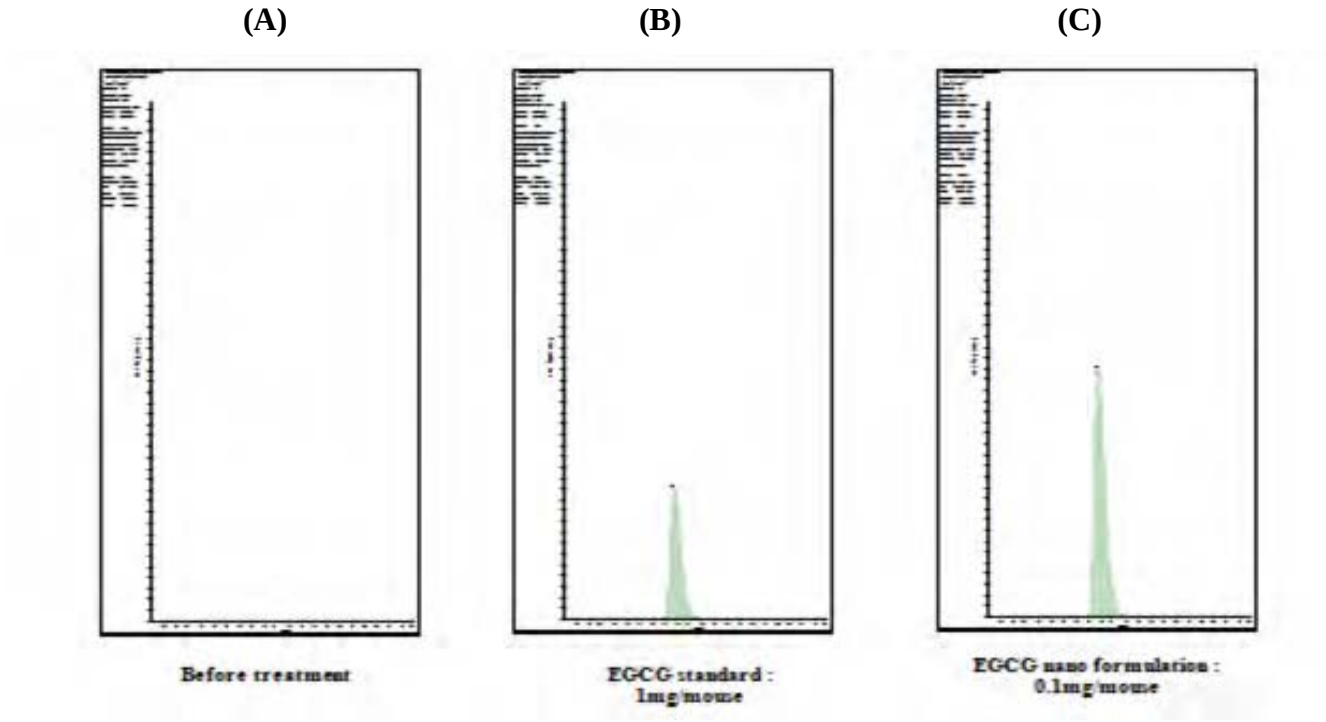


Figure 4: The LC/MS chromatograms of the selected fragment ion of (A) blank serum without EGCG (B) serum EGCG (m/z: 457/168.9; retention time: 1.77 min) from mouse treated with EGCG standard (1 mg) and (C) EGCG nano-formulation (0.1 mg), separately. The mouse blood was collected 2 hours post dosage.

NanoEGCG pharmacokinetics: Oral administration is the most favored route for delivery of chemopreventive agents. However, many agents have low bioavailability because of their poor biopharmaceutical and/or pharmacokinetic profile. As a result, large oral dose is required with conventional delivery systems to attain and maintain the desired levels. However, in majority of such cases high oral dose leads to adverse effects. Various drug delivery systems, each one having its own limitations, have been developed to overcome the hurdles of bioavailability and toxicity. Polymeric nanoparticles offer a great promise for drug delivery and in line with this fact we have successfully developed an oral formulation of nanoEGCG. Therefore, we investigated the pharmacokinetics of oral nanoEGCG in mice to determine the time course of EGCG disappearance from the circulation. **Study design:** Twenty four athymic nude mice were subjected to overnight fasting (39-40), divided into two groups and dosed next morning with either 3 mg/Kg body weight of EGCG or nanoEGCG by oral intubation. Blood was collected either from the retro-orbital plexus or mandible prior to and at 0.5, 1, 2, 4, 8, 16, 24, 48 and 96 hours post intubation. The pharmacokinetics of EGCG disappearance was determined by HPLC analysis of the serum separated from the blood.

Chitosan nanoparticles encapsulating EGCG can be synthesized by gelation methods. The nanoparticle size can be manipulated by different variables like the amount of chitosan, amount of salt. The desired size, which is less than 200 nm, was obtained with the method as mentioned above. The shape and morphology study by transmission electron microscope is in progress. An HPLC method was developed to determine the amount of EGCG encapsulated in the nanoparticles. HPLC analysis showed that chitosan nanoparticles can efficiently entrap EGCG.

Also we have developed a method to extract EGCG from the plasma with a recovery value more than 95%. With the help of an internal standard, the recovered amount of EGCG was measured by LC-MS/MS. The LOD of 0.05 ng and the LOQ of 1 ng/ml indicates the high sensitivity of our method. Figure 4 shows representative LC/MS chromatograms used to calculate the quantity of EGCG in serum by using the calibration curves from Figure 3.

The pharmacokinetics study is under progress. Since this is a time-bound study, blood was collected at selected time-points and the experiment is being repeated and the data analysis is in progress. Other parameters that might need to be optimized in nano-formulation are chitosan, the phosphate salt, and the size of the nanoparticles. These experiments are in progress.

Athymic nude mice tumor study:

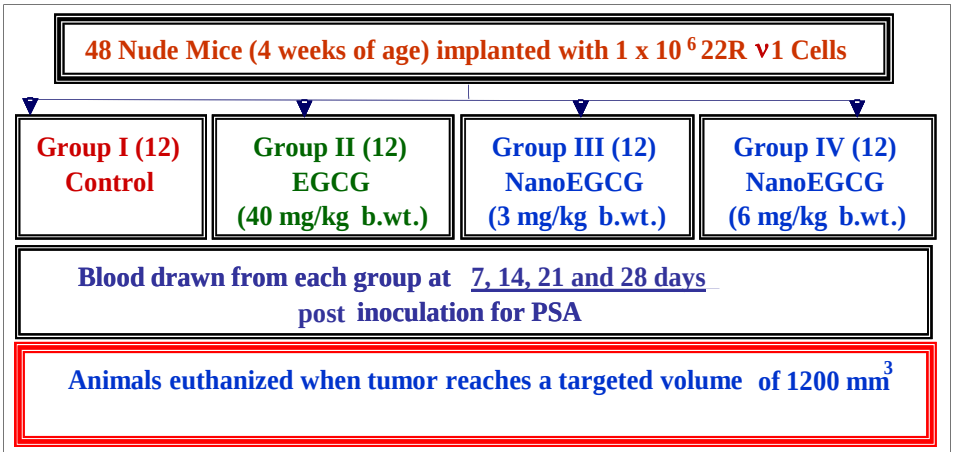
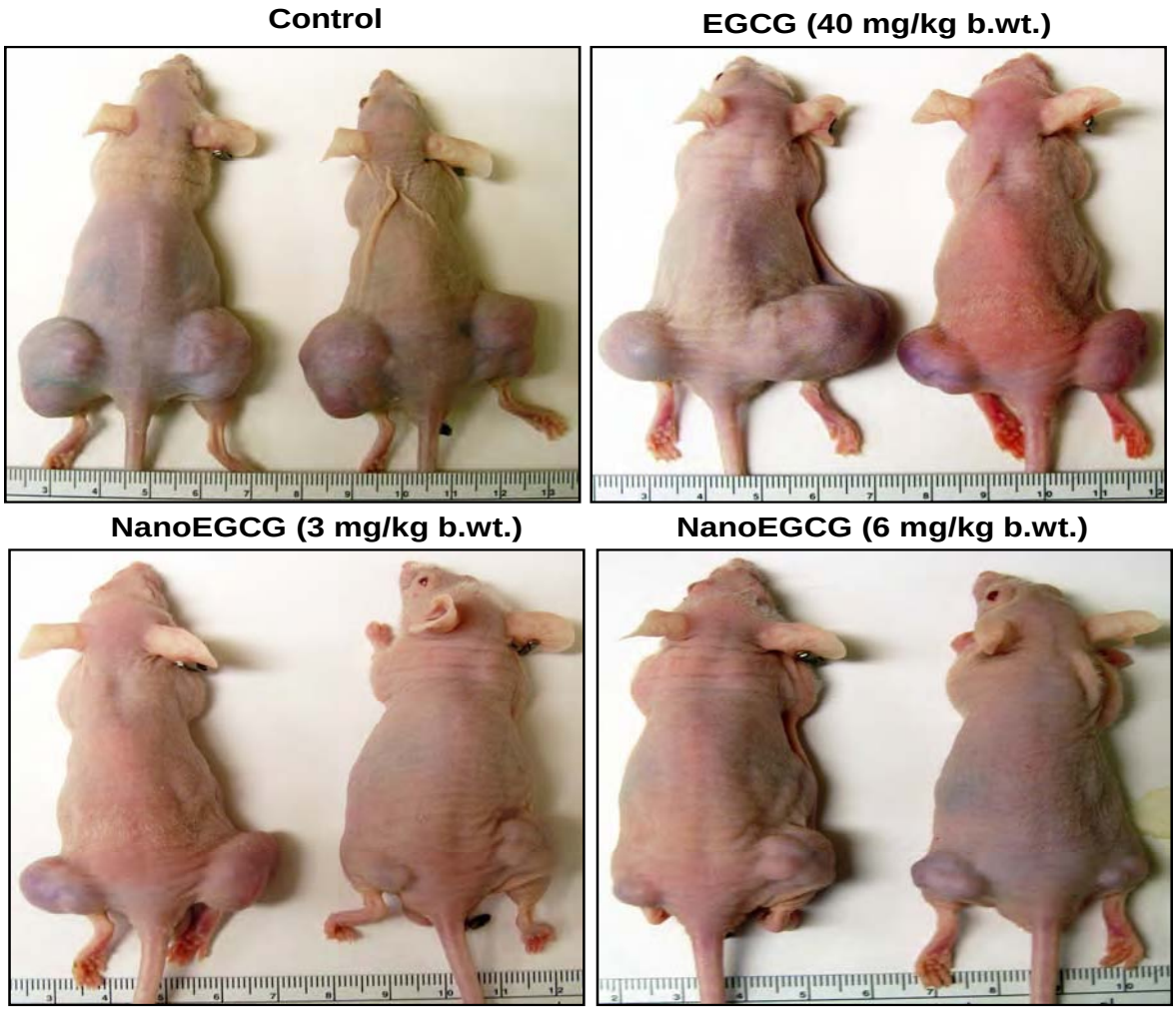


Figure 5 **Schematic representation of tumor studies in athymic nude mice**

We determined the effect of oral nanoEGCG on the growth of tumors in an *in vivo* situation utilizing athymic nude mice implanted with 22Rv1 cells that form reproducible tumors and also secrete PSA. **Study Design**: Forty eight male athymic nude mice (4 weeks of age) were housed four/cage and fed *ad libitum* with autoclaved semi-purified, AIN-76 B-40 diet. A total of 1 million 22Rv1 cells (in 50 µl DMEM + 50 µl Matrigel) were implanted by a sub-cutaneous injection on left and right sides, below the shoulders (2 tumors per mouse). **Treatment of nude mice**: Mice implanted with cells were randomly distributed into four groups of twelve each (**Figure 5**). Group I received void nano particles and served as the control. Group II received EGCG (40 mg/kg b.wt). Group III received nanoEGCG (3 mg/kg b. wt) and Group IV received nanoEGCG (6 mg/kg b. wt). Treatments were by oral intubation and started one day post cell inoculation, 5 times a week (Monday-Friday) until tumors reached a targeted volume of 1200 mm^3. **Tumor volume estimation**: The tumor size was measured by determining two perpendicular dimensions with calipers, and the volume was calculated using the formula $(a \times b^2)/2$, where a is the longer and b is the smaller dimension. The animals were also be evaluated for body weight, consumption of food and apparent signs of toxicity. At weekly intervals phlebotomy was performed to obtain sera for PSA estimation by ELISA.

NanoEGCG inhibits the growth of human prostate carcinoma CWR22Rv1 cells in athymic nude mice:

(A)



(B)

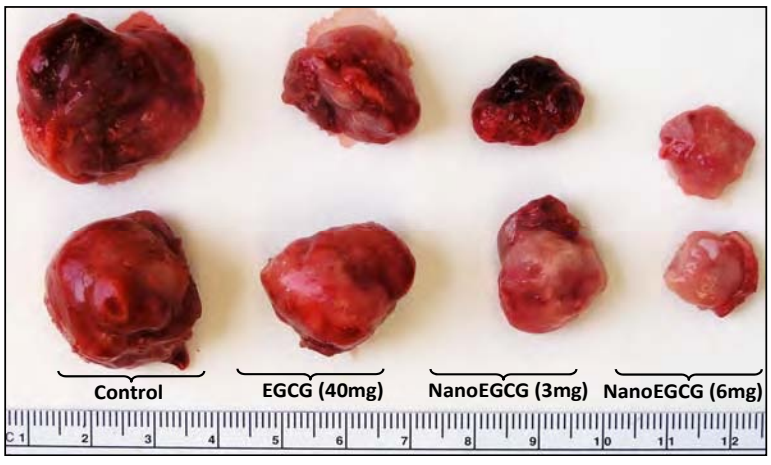


Figure 6

The treatment of athymic nude mice with nanoEGCG resulted in inhibition of AR-positive CWR22Rv1 tumor xenograft growth. The appearance of small solid tumors was observed in animals of control group 11 days after cell inoculation. This latency period was prolonged to 18 days in animals receiving EGCG (40 mg/kg body wt.) and 25 days in animals receiving nanoEGCG

(3 and 6 mg/kg body wt.). There was significant reduction in growth of prostate tumors in EGCG and nanoEGCG-treated animals as compared to control group (Figures 6-8). As depicted in Fig. 7, tumor growth, as inferred by computed tumor volume, was significantly inhibited in mice receiving EGCG and nanoEGCG. In control group, the average tumor volume of 1,200 mm^3 was reached in 32 days after tumor cell inoculation. At this time point, the average tumor volume was 514, 310 and 216 in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively. The average tumor volume of 1,200 mm^3 was achieved 46, 53 and 60 days after tumor cell inoculation in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively (Figures 7 and 8). There was dose-dependent inhibition of tumor growth by nanoEGCG and it was found to be more effective than EGCG.

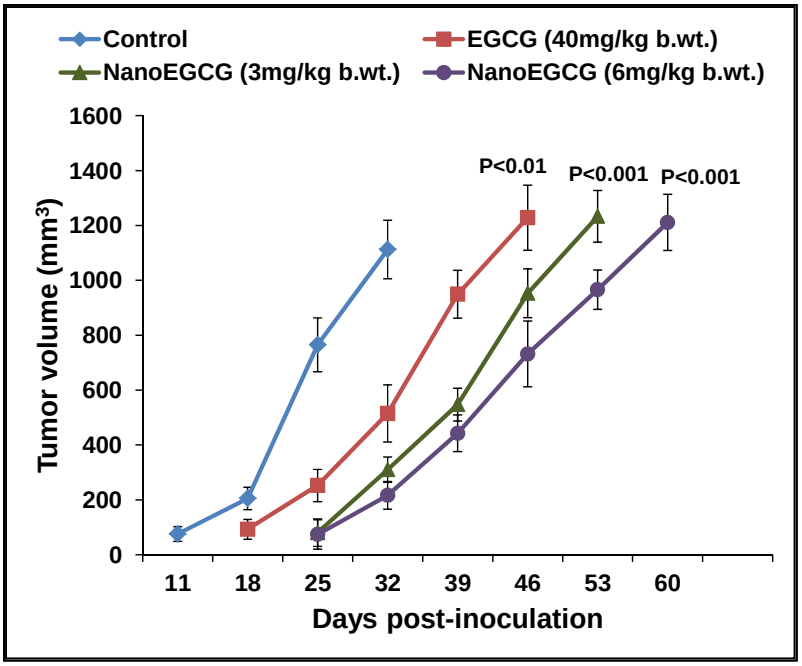


Figure 7

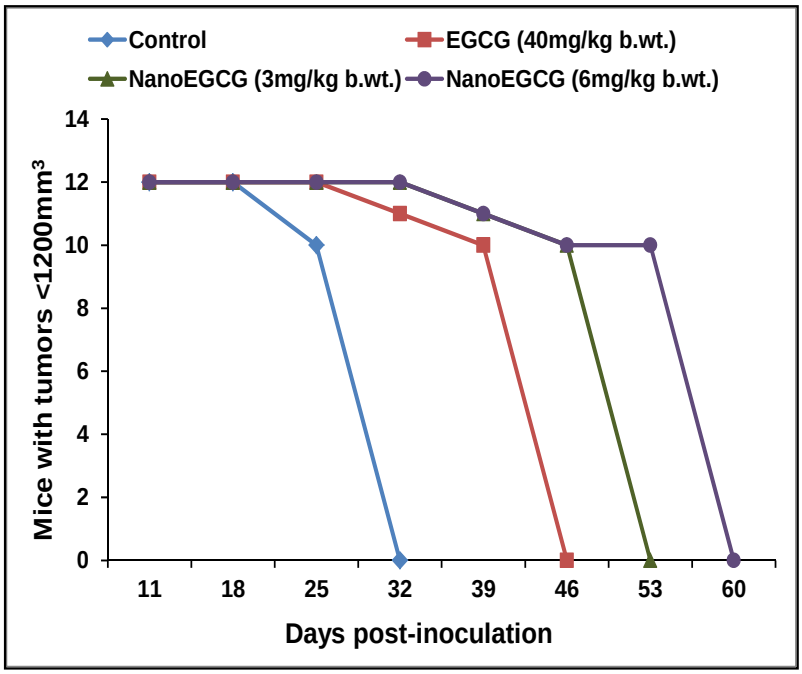


Figure 8

NanoEGCG inhibits PSA secretion in athymic nude mice:

During the course of tumor growth in animals at day 2, 8, 14, 20 and 26 after inoculation, blood was collected through the mandibular bleed. Quantitative sandwich ELISA was used to determine circulating PSA levels in mouse serum secreted by CWR22Rv1 tumor xenografts. PSA is a serine protease with highly prostate-specific expression and is the most widely employed marker in the detection of early prostate cancer. For these reasons, it is considered that agents which could reduce PSA levels may have important clinical implications for prostate cancer. It is currently the most accepted marker for assessment of prostate cancer progression in humans and is being detected in the serum of patients with prostate diseases including prostatitis, benign prostatic hypertrophy, and prostate cancer (4). In our study, we found that there was significant inhibition of secreted PSA levels by 13-36%, 26-54% and 57-72% in EGCG, nanoEGCG (3mg/kg body wt.) and nanoEGCG (6mg/kg body wt.) treated groups, respectively as compared to the control group normalized to tumor volume (Figure 9). Hence, our results show that treatment of mice with nanoEGCG caused dose-dependent significant decrease in the serum PSA in athymic nude mice and the effect was more pronounced with nanoEGCG than EGCG.

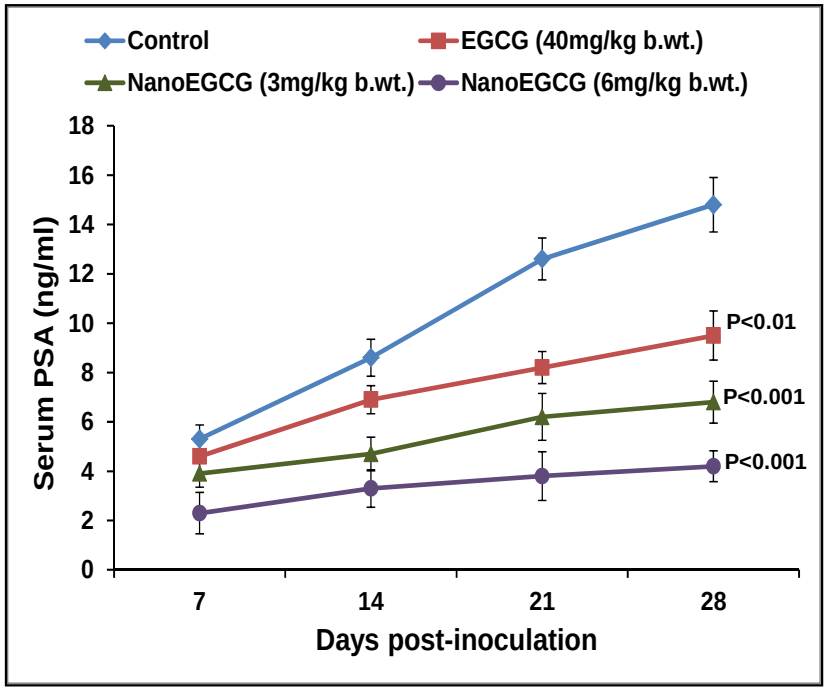


Figure 9

KEY RESEARCH ACCOMPLISHMENTS:

Prostate cancer remains the most common cancer in men in the United States and next only to lung cancer, is the second leading cause of cancer-related deaths in American males.

- We have been successful in developing an oral formulation of nanoEGCG using a naturally occurring polymer chitosan which we observed to result in a steady and sustained release of EGCG in the plasma of mice. This nano formulated green tea has a significant longer half-life compared to non-encapsulated EGCG.
- Through these studies in athymic nude mice, we established the anti-tumor efficacy of oral nanoEGCG vs. EGCG alone. Oral formulated nanoEGCG compared to EGCG resulted in a significant inhibition of tumors in nude mice based on increased bioavailability.
- We also observed a significant dose-dependent decrease in serum-PSA levels in athymic nude mice in nanoEGCG-treated groups.
- Far superior tumor growth inhibitory effects were observed with oral nanoEGCG at significantly lower doses.

- Our experimental design established a dose dependent efficacy of oral nanoEGCG which will guide us on the dose required for further studies in Nkx3.1/Pten and TRAMP mice.
- In future studies, we will investigate if supplementation of oral nanoEGCG will inhibit the development of PIN lesions in the Nkx3.1/Pten mutant mice via modulations in i) pro-inflammatory milieu, and ii) oxidative stress in the prostate. Further, we will determine the preventive and therapeutic efficacy of oral nanoEGCG and to identify the stage of prostate cancer development that is most vulnerable to the anti-cancer effects of oral nanoEGCG in the transgenic TRAMP mice.

REPORTABLE OUTCOMES:

None to report for this period.

CONCLUSION:

The issues related to bioavailability and perceived toxicity of green tea pose a great challenge which are addressed in this study through proposing the use of the novel and uniquely formulated oral nanoEGCG. Nanotechnology will offer significant and increasing improvements in options not only for therapeutic interventions against malignancies but also for disease prevention, a concept that is being addressed through this proposal. It is expected that nanotechnology will continue to have a profound and positive impact on human health. Many nanotechnology-based diagnostic and treatment modalities already are in use, with many others at various stages of pre-clinical and clinical testing. Several nanotechnology platforms hold great promise for diagnosis and treatment of cancer. We propose that nanotechnology will serve as a cornerstone in cancer chemoprevention. Through this study, we propose a novel preventive and therapeutic modality using EGCG that will address issues related to bioavailability, toxicity and dose requirement that are major reasons for its limited success in humans.

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APPENDICES:

None.